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Mixed chimerism

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Induction of mixed chimerism has the potential to overcome the current limitations of transplantation, namely chronic rejection, complications of immunosuppressive therapy and the need for xenografts to overcome the current shortage of allogeneic organs. Successful achievement of mixed chimerism had been shown to tolerize T cells, B cells and possibly natural killer cells, the lymphocyte subsets that pose major barriers to allogeneic and xenogeneic transplants. Current understanding of the mechanisms involved in tolerization of each cell type is reviewed. Considerable advances have been made in reducing the potential toxicity of conditioning regimens required for the induction of mixed chimerism in rodent models, and translation of these strategies to large animal models and in a patient are important advances toward more widespread clinical application of the mixed chimerism approach for tolerance induction.

Keywords: tolerance; mixed chimera; bone marrow transplantation; xenotransplantation; natural antibodies; natural killer cells

1. INTRODUCTION

Chronic rejection and complications of immunosuppressive therapy currently limit the success of allotransplants, providing the impetus for developing strategies for specific tolerance induction. Another impetus for the induction of tolerance arises from the current shortage of allogeneic organs, which has increased interest in the use of other species as xenograft donors. However, immune barriers to xenografts may be even greater than those to allografts (Auchincloss 1995), and the induction of both B- and T-cell tolerance, as well as natural killer (NK) cell tolerance, may be essential to the ultimate success of xenotransplantation. Studies discussed below suggest that the induction of mixed chimerism has the potential to achieve all of these goals.

2. CHIMERISM AND TOLERANCE

The word 'chimerism' is used in this review to describe the existence in a recipient of haemopoietic elements from a donor that is allogeneic or xenogeneic to the recipient. In our terminology, 'microchimerism' refers to chimerism that is not measurable by flow cytometry (FCM) (which usually has a detection limit in the range of 0.1–1%), and requires sensitive techniques, such as polymerase chain reaction (PCR), for its detection. 'Mixed chimerism', the topic of this review, refers to a state in which donor and host haemopoietic elements of multiple lineages coexist at levels detectable by FCM. 'Full chimerism', on the other hand, is a state in which essentially all haemopoietic elements are derived from a donor stem cell inoculum. Although it has been suggested that microchimerism may lead to tolerance in humans and animal models (Starzl *et al.* 1992), there is considerable evidence against this concept, as well as some evidence to suggest that early microchimerism may play a role in inducing but not maintaining tolerance in certain models (Bushell *et al.* 1995; Ko *et al.* 1999). The presence of microchimerism failed to predict tolerance in a neonatal tolerance model (Alard *et al.* 1995) and was not always observed in tolerant rat recipients of cardiac allografts (Fisher *et al.* 1996). Thus, it has become clear that microchimerism neither predicts tolerance nor is required to maintain tolerance under all circumstances. We shall not consider microchimerism further, but will instead focus on mixed chimerism, a state which has been clearly shown to have the capacity to reliably induce a robust state of donor-specific tolerance.

The pioneering work of Owen, Medawar and others, beginning 50 years ago, led to the observation that haemopoietic chimerism can be associated with a state of donor-specific tolerance (reviewed in Charlton *et al.* 1994). Tolerance can most readily be induced by allogeneic haemopoietic cells in animals that are developmentally immunodeficient, or in which immunodeficiency has been artificially induced. As is discussed in more detail below (§4(a)), one major mechanism by which haemopoietic cells induce tolerance is through their ability to cause intrathymic clonal deletion of thymocytes with T-cell receptors that recognize antigens expressed by the haemopoietic cells (Marrack *et al.* 1988). When pre-existing peripheral T cells are adequately eliminated and allogeneic or xenogeneic bone marrow (BM) engraftment is achieved, tolerance to the most immunogenic allografts, such as fully major histocompatibility complex (MHC)-mismatched skin grafts and small bowel grafts, is reliably attained (Sharabi & Sachs 1989; Orloff *et al.* 1994). As is discussed below (§4(a)), BM also has the capacity to tolerize pre-existing peripheral T cells under certain

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Table 1. *Barriers to allogeneic and xenogeneic haemopoietic cell engraftment and haemopoiesis*

physiological or regulatory
space and physical niches
cytokines (soluble and stromal cell-bound)[a]
adhesion molecules and extracellular matrix proteins[a]
immunological
T cells (CD4 and CD8; CD4CD8 γδ T cells[a])
antibodies (from prior sensitization; NAbs[a])
NK cells[a]

[a] May be an especially important barrier to xenogeneic haemopoiesis.

conditions, avoiding the requirement for complete depletion of recipient T cells and the consequent period of marked immunosuppression before regeneration of the T-cell repertoire can take place.

3. OBSTACLES TO THE USE OF HAEMOPOIETIC CELL TRANSPLANTATION FOR THE INDUCTION OF CENTRAL TOLERANCE IN MAN

In view of its potent and reliable ability to induce intrathymic tolerance, it may seem surprising that haemopoietic cell transplantation has not yet been applied to the induction of tolerance in man. However, there are numerous immunological and physiological obstacles to the engraftment and function of allogeneic and xenogeneic haemopoietic cells. These are summarized in table 1. To ensure lifelong deletional tolerance, donor cells with long-term multilineage repopulating ability, known as pluripotent haemopoietic stem cells (PHSCs), must survive and function sufficiently to provide a life-long source of donor antigens in the thymus. The simplest and most widely used approach to overcoming both the immunological and the physiological barriers to marrow engraftment in adult animals has been to pre-treat recipients with lethal total-body irradiation (TBI) prior to BM transplantation (BMT). While this procedure is well tolerated and reliably induces chimerism and tolerance in rodents, its use is unfortunately less feasible in humans. Lethal TBI and chemotherapy are unduly toxic for use in patients who do not have malignant diseases and who may have a higher likelihood of survival without BMT. Furthermore, BMT across human leucocyte antigen (HLA) barriers is associated with an inordinately high risk of graft-versus-host disease (GVHD) and engraftment failure (Clift & Storb 1987; Anasetti *et al.* 1989; reviewed in Martin 1994). The latter complication is potentially fatal in recipients of supralethal doses of TBI and chemotherapy, and this complication is exacerbated when T-cell depletion of the donor graft is performed to prevent GVHD (Martin *et al.* 1988; Bordignon *et al.* 1989; Kernan *et al.* 1987). These observations suggest that studies in mice showing that sublethal irradiation, with or without cyclophosphamide treatment, is sufficient to allow engraftment of allogeneic marrow given in conventional doses (Pierce *et al.* 1985; Colson *et al.* 1995), are unlikely to be directly applicable to HLA-mismatched allogeneic or xenogeneic transplantation in humans.

It has been suggested by a number of studies that donor T lymphocytes lacking the capacity to react to host alloantigens (and hence lacking the potential to induce GVHD) may nevertheless enhance donor engraftment (Sykes *et al.* 1988*a*, 1989; Lapidot *et al.* 1990; Fowler *et al.* 1998; Nakamura & Gress 1990; Martin 1996). A veto mechanism has been implicated in this phenomenon. A veto phenomenon has also been suggested to explain the capacity of $CD8^+CD3^-$ donor BM cells (DBMCs) to promote kidney allograft survival in monkeys treated with anti-thymocyte globulin (ATG) (Thomas *et al.* 1991, 1994; Asiedu *et al.* 1999). In addition, a $CD3^+$, T-cell receptor (TCR) $\alpha\beta^-$, TCR$\gamma\delta^-$, $CD8^+$ BM cell (BMC) population expressing a novel 33 kDa glycoprotein in association with TCR β-chain (Schuchert *et al.* 2000) has been reported to facilitate donor PHSC engraftment (Kaufman *et al.* 1994). Other studies have suggested that a $CD8\alpha^+$ non-T-cell lymphoid dendritic-cell-like population and a classical $CD8^+$ T-cell population are the major graft-facilitating cells in marrow (Gandy *et al.* 1999).

A recent advance in achieving engraftment of extensively HLA-mismatched, T-cell-depleted stem cells without GVHD has involved the use of high stem cell doses in lethally conditioned recipients (Aversa *et al.* 1994, 1998). Thus far, however, the major limitation to the success of this approach has been a high incidence of infectious complications in association with markedly delayed immunological recovery. Before the stem cell transplantation approach to tolerance can be applied clinically, therefore, it will be necessary to understand the immunological and physiological barriers to haemopoiesis in allogeneic and xenogeneic environments, and to use this information to develop highly specific and relatively non-toxic methods of overcoming these barriers.

A major concern with using BMT to induce tolerance to fully MHC-mismatched cadaveric donors has involved the immunoincompetence that occurs as a consequence of the full MHC disparity between the positive selecting elements in the thymus, which are of host origin, and the antigen-presenting cells (APCs) in the periphery, which are entirely of donor origin in full chimeras. These T cells are ineffective at mounting relevant donor-restricted immune responses (Singer *et al.* 1981; Zinkernagel *et al.* 1978, 1980; Ildstad *et al.* 1985) due to the lack of host APCs that could induce generation of host-restricted responses. However, this problem could be overcome by creating mixed chimerism of host-type and allogeneic haemopoietic cells. Mixed chimeras have a lifelong source of host APCs that allow effective generation of host-restricted immune responses (Singer *et al.* 1981). Ildstad and colleagues (Ildstad & Sachs 1984; Ildstad *et al.* 1985) showed that mixed allogeneic chimeras produced by reconstituting lethally irradiated mice with a mixture of T-cell-depleted allogeneic and host-type marrow were specifically tolerant of donor-tissue grafts, with full immunocompetence that was superior to that of full allogeneic chimeras (Singer *et al.* 1981; Zinkernagel *et al.* 1978, 1980; Ildstad *et al.* 1985; Ruedi *et al.* 1989). Murine mixed chimeras created with high doses of total lymphoid irradiation provided an even earlier demonstration that mixed chimerism leads to a state of donor-specific tolerance (Slavin *et al.* 1977, 1978*a*,*b*; Vallera *et al.* 1982).

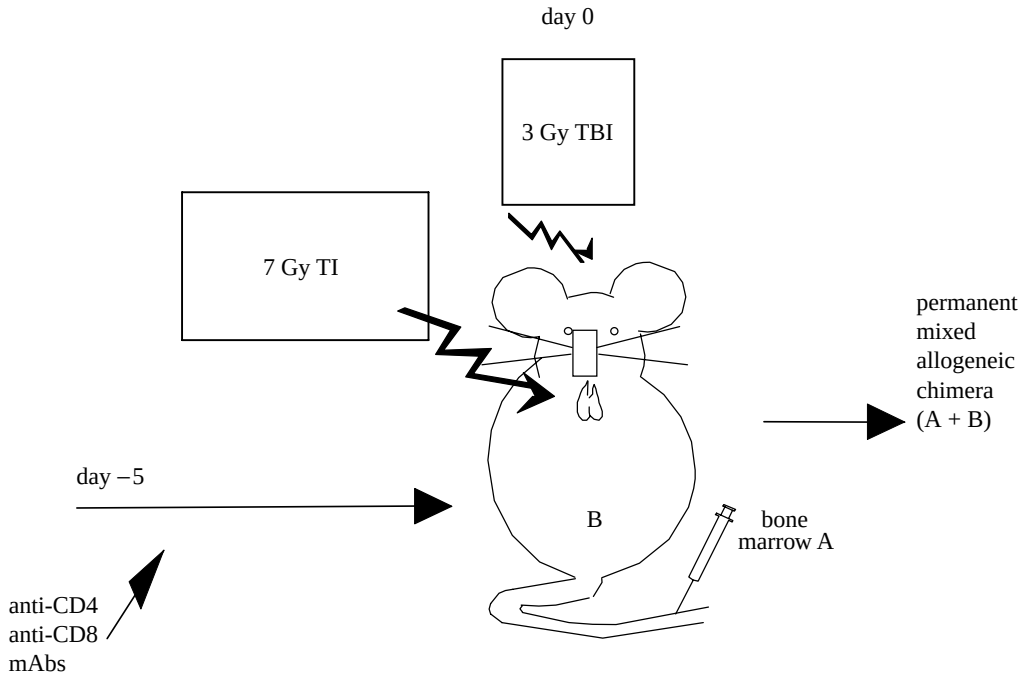

Figure 1. Method for producing mixed allogeneic (fully MHC mismatched) chimeras using a non-myeloablative conditioning regimen. Depleting anti-mouse mAbs are: anti-CD4, GK1.5 (Dialynas *et al.* 1983); anti-CD8 mAb, 2.43 (Sarmiento *et al.* 1980).

4. IMMUNOLOGICAL BARRIERS TO ALLOGENEIC AND XENOGENEIC HAEMOPOIETIC CELL ENGRAFTMENT

(a) *T-cell barriers to BM engraftment*

A model that allows induction of mixed chimerism and donor-specific tolerance across MHC barriers in mice without myeloablative conditioning (Sharabi & Sachs 1989) has helped to define the immunological barriers to marrow engraftment. This model, which is illustrated in figure 1, involves the use of depleting anti-CD4 and anti-CD8 monoclonal antibodies (mAbs) followed by a low dose (3 Gy) of TBI and a higher dose (7 Gy) of local thymic irradiation (TI). Similar results have recently been achieved using anti-TCR$\alpha\beta$ antibody and 3 Gy TBI (Nomoto *et al.* 1995). These (Sharabi & Sachs 1989; Sharabi *et al.* 1992) and other studies (Vallera *et al.* 1994) have shown that either $CD4^+$ or $CD8^+$ T cells of the host can readily reject fully MHC-mismatched marrow, and as expected, that $CD4^+$ cells reject class II mismatched marrow, and CD8 cells reject class I disparate marrow. Additionally, CD8 cells also play a significant role in rejecting class II mismatched marrow, and $CD4^+$ host cells pose a weak but measurable barrier to class I mismatched marrow (Sharabi *et al.* 1992; Vallera *et al.* 1994).

To fully overcome the T-cell barrier to alloengraftment, both T cells in the peripheral lymphoid tissues and those in the thymus must be considered. If the pre-existing peripheral alloreactivity is not adequately eliminated, eventual rejection may ensue even if initial intrathymic chimerism is achieved. Likewise, a failure to completely eliminate intrathymic alloreactive T cells can result in a situation in which initial peripheral chimerism is achieved but subsequently lost due to the later emigration of non-tolerant thymocytes maturing in the absence of donor antigen, which can eliminate donor cells intrathymically and extrathymically (Sharabi & Sachs 1989; Tomita *et al.* 1996*a*,*b*; Nikolic *et al.* 2001).

Induction of lasting chimerism with the method shown in figure 1 reflects the fact that peripheral alloreactivity is overcome prior to BMT by using T-cell-depleting mAbs, and intrathymic allroeactivity is eliminated by TI of the host (Sharabi & Sachs 1989; Nikolic *et al.* 2001). Subsequently, newly developing host and donor thymocytes encounter both donor and host APCs in the thymus, and this results in the deletion of thymocytes recognizing donor or host antigens. Such donor- and host-reactive T cells are consequently absent from the spleen and lymph nodes, where pre-existing mature T cells have been eliminated by the T-cell-depleting antibodies in the conditioning. This specific deletion of donor-reactive T cells is evident throughout the lymphoid system for the life of the animal (Tomita *et al.* 1994*a*). Clonal deletion has been demonstrated both by analysis of T cells bearing Vβ subunits that recognize superantigens presented by donor MHC, and by the use of recipients with transgenic TCR specific for a transplantation antigen. In hosts with a transgenic TCR (2C) specific for a donor class I antigen, H-$2L^d$, central deletion of donor-reactive (anti-$H2L^d$) CD8 cells was shown by the disappearance of CD8 single-positive thymocytes expressing the transgenic TCR (Manilay *et al.* 1998*a*). Donor class II^+ cells with histological morphology resembling dendritic cells or macrophages can be found intrathymically in mixed chimeras throughout their lives, beginning as early as a few days after BMT (Tomita *et al.* 1994*a*; Manilay *et al.* 1998*a*), and these cells are believed to be the mediators of negative selection in these animals. Host cells with a similar phenotype are also present in abundance, resulting also in the intrathymic deletion of host-reactive T cells (Tomita *et al.* 1994*a*). In mice receiving allogeneic BMT after

conditioning with the regimen shown in figure 1, the first wave of recovering thymocytes (day 10) was completely deleted of mature host thymocytes recognizing superantigens presented by donor I-E MHC molecules, despite the presence of only very small numbers of donor I-E-bearing cells in the thymus at this early time (Tomita *et al.* 1994*a*). The role of central deletion as the only significant mechanism by which tolerance is maintained in this non-myeloablative model is also supported by the lack of evidence for anergy or suppression. Anergy was ruled out by studies in which established chimeras were thymectomized before donor antigen was removed by administration of an mAb specific for donor MHC (Khan *et al.* 1996). Tolerance persisted in these mice despite the absence of donor antigen, which would be required for maintenance of tolerance through anergy. Active suppression does not play a major role either, as chimerism and tolerance were easily broken in established chimeras by the infusion of naive host-type spleen cells or by the removal of antigen when the host thymus was left intact so that non-tolerant T cells could be subsequently generated in the thymus (Khan *et al.* 1996).

Recently, a number of modifications have been made to the conditioning regimen in figure 1 to make it even less toxic and hence more attractive to transplantation clinicians. These include the replacement of TI with a second injection of depleting anti-T-cell mAbs (Tomita *et al.* 1996*a*,*b*), the omission of TBI by administering very high marrow doses (Sykes *et al.* 1997), the omission of both TI and host T-cell-depleting mAbs using co-stimulatory blockade (Wekerle *et al.* 1998), and the replacement of TI with a single injection of one co-stimulatory blocker (Wekerle *et al.* 1999). Most recently, we have achieved lasting chimerism and donor-specific tolerance with a regimen that requires no host pre-conditioning, by giving a high dose of fully MHC-mismatched donor marrow followed by a single injection of each of two co-stimulatory blockers (Wekerle *et al.* 2000). This regimen is shown in figure 2. Other protocols, using different forms of myelosuppression and T-cell elimination, have been developed recently for the induction of mixed chimerism in rodents (Mayumi & Good 1989; Nomoto *et al.* 1995; Colson *et al.* 1995; De Vries-van der Zwan *et al.* 1994, 1997, 1998; Koch & Korngold 1997; Hale *et al.* 2000).

The mechanisms of tolerance in animals receiving BMT under cover of co-stimulatory blockade instead of T-cell depletion are currently under active investigation. Since large numbers of alloreactive T cells are present in the peripheral lymphoid tissues of these animals, peripheral tolerance mechanisms must be involved. Specific deletion of donor-reactive cells appears to play a role in this tolerance (Wekerle *et al.* 1998, 2000). Both activation-induced cell death and the type of 'passive cell death' associated with cytokine withdrawal appear to play a role in this deletion (Wekerle *et al.* 2001). However, donor-specific tolerance can be shown to be complete in mixed lymphocyte reactions (MLRs) by one week post-transplantation, when deletion of donor-reactive CD4 cells is only partial (Ito *et al.* 2001), suggesting that mechanisms in addition to deletion are involved in the early tolerization of peripheral CD4 cells by donor BM in the presence of co-stimulatory blockade. Evidence has been obtained for a role for CD28, but there is no role for

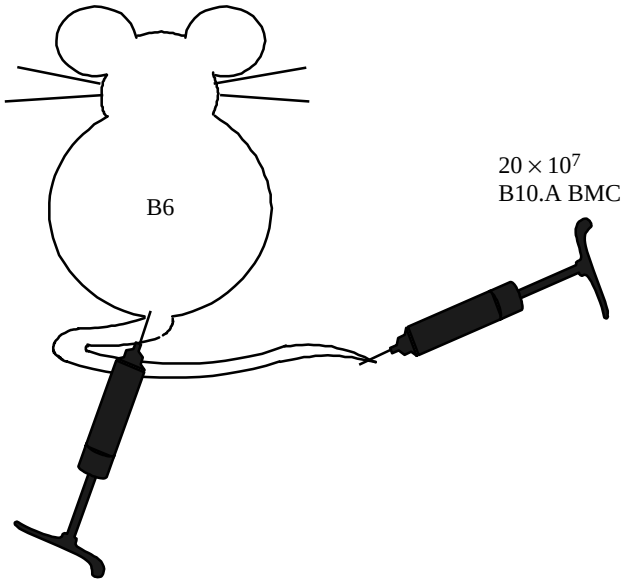

Figure 2. Method for inducing mixed chimerism across a full MHC barrier with high-dose marrow in mice receiving no preconditioning, and no myelosuppressive drugs or irradiation. Lasting chimerism is associated with donor-specific skin graft tolerance and systemic tolerance, as measured by donor-specific unresponsiveness in MLR and CML assays. Intrathymic deletion of donor-reactive host thymocytes is a major mechanism maintaining long-term tolerance in these mice (Wekerle *et al.* 2000).

CD40 ligand (CD40L)-mediated signals to the CD4 T cells for the induction of tolerance with this approach (Kurtz *et al.* 2001).

Although attractive because they do not require host T-cell depletion, one limitation to the approaches involving co-stimulatory blockade in place of peripheral T-cell depletion to achieve allogeneic BM engraftment is that it is not 100% successful, i.e. with each regimen, there is always a fraction of animals that do not achieve permanent chimerism or donor-specific skin graft acceptance (Wekerle *et al.* 1998, 2000). Recently, we have observed that the use of either cytotoxic T-lymphocyte antigen 4 immunoglobulin (CTLA-4-Ig) or an anti-CD154 (CD40L) mAb overcame the requirement for TI or repeated injection of T-cell-depleting antibodies in mice receiving a T-cell depletion regimen that is in itself insufficient to permit induction of lasting chimerism. The combination of co-stimulatory blockade and inadequate T-cell depletion very reliably leads to stable, lasting chimerism and donor-specific tolerance (Wekerle *et al.* 1999). Most recently, we have observed that CD4 T cells need not be eliminated at all, i.e. that a single injection of anti-CD40L mAb is sufficient to allow BMT to induce tolerance of CD4 cells in mice receiving an injection of depleting anti-CD8 mAb (Ito *et al.* 2001).

GVHD does not occur in the rodent models described above, despite the use of unseparated DBMCs. This is most readily explained by the continued presence of the T-cell-depleting or co-stimulatory blocking antibodies in the serum of the hosts at the time of BMT (Tomita *et al.* 1996*a*), but it may also reflect an inherently reduced susceptibility of mixed chimeras to GVHD (Sykes *et al.* 1988*b*,*c*; Ildstad *et al.* 1986).

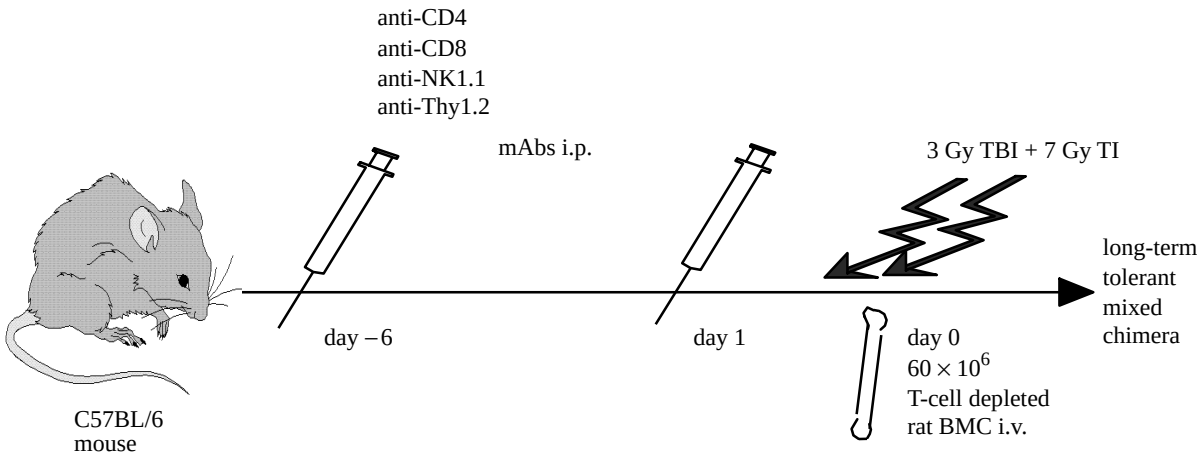

Figure 3. Method for producing mixed xenogeneic (rat→mouse) chimeras using a non-myeloablative conditioning regimen. Anti-mouse CD4 mAb is GK1.5 (Dialynas *et al.* 1983), anti-mouse CD8 mAb is 2.43 (Sarmiento *et al.* 1980), anti-Thy1.2 mAb is 30H-12 (Ledbetter & Herzenberg 1979) and anti-NK1.1 mAb is PK136 (Koo & Peppard 1984). Rat marrow was T-cell depleted as described (Sharabi *et al.* 1990).

The T-cell-depleting mAb-based non-myeloablative approach to host conditioning used in the mouse allogeneic BMT model shown in figure 1 has been extended to a xenogeneic combination, rat→mouse (figure 3). However, in order to achieve success with this approach in a xenogeneic combination, it was necessary to pretreat the hosts with mAbs against NK1.1 and Thy1.2 in addition to anti-CD4 and anti-CD8 mAbs (Sharabi *et al.* 1990). We have recently demonstrated that the requirement for host treatment with anti-NK1.1 mAb reflects the capacity of host NK cells to reject rat marrow (see § 4(b)), whereas the need to treat hosts with anti-Thy1.2 reflects a role for γδ T cells in resisting rat marrow engraftment in mice (Nikolic 2001). These studies demonstrate the existence of several components of the immune system that resist xenogeneic, but not allogeneic, marrow engraftment. In this model, the rat donor marrow must be T-cell depleted *ex vivo* in order to prevent GVHD, since the anti-T-cell mAbs used to pretreat the host are specific for murine T-cell antigens, and do not deplete the donor rat T cells *in vivo*.

(b) *The NK cell barrier to allogeneic and xenogeneic marrow engraftment*

In short-term studies measuring myeloid progenitor cell proliferation *in vivo*, NK cells have been shown to resist allogeneic marrow engraftment (Cudkowicz & Bennett 1971). While NK-cell-mediated resistance can be reproducibly measured in these assays, these cells actually present only a relatively weak barrier to the engraftment of allogeneic PHSCs (Lee *et al.* 1996; Aguila & Weissman 1996). In fact, it is difficult to detect a significant effect of the NK-cell barrier to allogeneic BMT when 'standard' marrow doses are given to mice receiving the non-myeloablative regimen in figure 1 (Lee *et al.* 1996). Nevertheless, it is possible that these cells may pose a more significant barrier to HLA-mismatched human marrow engraftment, in which stem cell and progenitor cell numbers in the donor inoculum may be more limited (O'Reilly *et al.* 1986).

NK cells play a much more significant role in resisting the engraftment of xenogeneic than allogeneic marrow in mice (Sharabi *et al.* 1990; Nikolic *et al.* 2001). Since NK-cell-mediated resistance to the engraftment of class I deficient (β2-microglobulin knockout) murine marrow is much greater than that to allogeneic marrow (Bix *et al.* 1991), we have speculated (Manilay & Sykes 1998) that allogeneic marrow is partially protected from NK-cell-mediated resistance due to the expression of class I molecules that are recognized by NK-cell-surface inhibitory receptors that cross-react on multiple class I alleles (Hoglund *et al.* 1997). Such inhibitory receptors may not interact well across species barriers (Seebach *et al.* 1997; Seebach & Waneck 1998; Sasaki *et al.* 1999*a*,*b*), whereas at least some NK-cell-activating receptors do function between species (Nakamura *et al.* 1999; Idris *et al.* 1998). This could explain the greater susceptibility of xenogeneic than allogeneic haemopoietic cells to NK-cell-mediated rejection.

In view of their importance in resisting xenogeneic marrow engraftment and their lesser role in alloresistance, the question of whether or not NK cells are tolerized by induction of mixed chimerism is relevant to the stability of chimerism in BMT recipients. Given the stability of chimerism in mixed allogeneic chimeras prepared as in figure 1, we hypothesized that host and donor-derived NK cells would be tolerant to donor and host MHC antigens, respectively. Regulation of Ly-49 receptors, which recognize specific MHC class I ligands, is thought to play a role in the self-tolerance of NK cells in mice. We hypothesized that individual NK cells in mixed chimeras must be tolerant of both donor and host antigens, and that this would be associated with alterations in the expression of Ly-49 receptors. Changes in the levels of expression of Ly-49 receptors were indeed observed among donor and host NK cells of mixed chimeras. The presence of the MHC ligand for a given Ly-49 receptor invariably led to reduced levels of that receptor in mixed chimeras on cells of the mouse strain that lacked the ligand (Sykes *et al.* 1993; Manilay *et al.* 1998*b*, 1999). For example, B6 NK cells, regardless of whether they were of donor (B6→BALB/c mixed chimeras) or recipient (BALB/c→B6 chimeras) origin, showed reduced levels of expression of Ly-49A and Ly-49G2 compared with

control B6 NK cells whenever BALB/c cells, which express D^d and D^d/L^d ligands for Ly-49A and Ly-49G2, respectively, were present. Furthermore, in mixed chimeras with differing levels of donor chimerism, we observed a quantitative relationship between the level of downregulation of Ly-49A, Ly-49C and Ly-49G2 and the number of haemopoietic cells expressing their MHC ligands (Manilay *et al.* 1999). Collectively, our results suggest that Ly-49 receptor expression levels are determined by interactions with their ligands, which tend to downmodulate the receptors, and argue against the concept of receptor 'calibration' to a specific level needed for tolerance. In fact, *in vitro* functional assays to test whether host and donor NK cells were tolerant to each other showed that donor (BALB/c) NK cells from mixed chimeras efficiently lysed host (B6) targets, indicating that donor (BALB/c) NK cells developing in BALB/c→B6 mixed chimeras were not tolerant to host (B6) antigens. Similarly, host (B6) NK cells from mixed chimeras failed to show tolerance to the BALB/c donor. Similar results were seen in 'reverse' B6→BALB/c mixed chimeras (Manilay *et al.* 1998*b*). In all of these studies, however, NK cells from mixed chimeras did not kill syngeneic targets to any greater extent than was observed for NK cells from non-transplanted controls or normal mice of the same strain. Thus, tolerance to 'self', as defined by the MHC antigens expressed by the NK cell itself, appears to develop normally in the mixed chimeric environment. The lack of tolerance to non-self MHC suggests that an inhibitory receptor for allogeneic MHC may not be expressed on all NK cells in mixed chimeras.

In contrast to these *in vitro* findings, *in vivo* studies from several other groups have demonstrated NK cell tolerance in irradiation chimera models (Wu & Raulet 1997), and in mice with mosaic expression of an MHC transgene (Johansson *et al.* 1997; Rosenzweig *et al.* 1996), and these have not always been consistent with *in vitro* studies showing a lack of such tolerance (Chadwick & Miller 1992; Johansson *et al.* 1997). In these and our own *in vitro* assays, NK cells are activated by culture in interleukin 2 (IL-2), whereas NK cells were not intentionally activated in any of the *in vivo* assays. A tolerance mechanism that operates at the level of regulation of NK cell activation *in vivo* might explain the *in vivo* tolerance observed in models in which tolerance is not preserved in *in vitro* assays, when exogenous IL-2 is added. *In vivo* studies of NK cell tolerance in mixed chimeras prepared with the regimen in figure 1 provided clear evidence of *in vivo* tolerance to haemopoietic cells of the recipient and donor strains (Y. Zhao and M. Sykes, unpublished data), despite the above *in vitro* evidence to the contrary. These data, which are consistent with the observation that chimerism tends to remain stable over life in mixed chimeras, suggest that donor and host NK cells, while not tolerant to one another by the same mechanisms as they are tolerant to their own MHC antigens, are nevertheless functionally tolerant of one another *in vivo*.

It is not yet known whether or not similar functional tolerance of NK cells develops in mixed xenogeneic chimeras. However, chronic host NK depletion did not significantly delay the gradual loss of rat chimerism in mixed chimeras prepared with the regimen in figure 3 (Lee *et al.* 1995), suggesting that a failure of host NK cell tolerance to the donor is not the predominant reason for the loss of chimerism that occurs in animals that are tolerant of their donors at the T- and B-cell levels (Lee *et al.* 1995; Aksentijevich *et al.* 1992; Nikolic *et al.* 1998).

(c) *Potential of mixed chimerism to overcome the natural antibody barrier to xenogeneic marrow engraftment*

We have previously demonstrated that preformed natural antibodies (NAbs) constitute a significant barrier to xenogeneic BM engraftment in the concordant rat→mouse BMT model (Aksentijevich *et al.* 1991) and they are likely to do so in discordant xenogeneic systems as well (Yang *et al.* 1998). Pigs are widely believed to be the most suitable xenogeneic donor species for transplantation to humans (Sachs 1994). In humans and Old World monkeys, the major specificity recognized by NAb on porcine tissues is a ubiquitous carbohydrate epitope, Galα1-3Galβ1-4GlcNAc-R (αGal) (Good *et al.* 1992). Mixed chimerism was shown in the non-myeloablative rat-to-mouse BMT model in figure 3 to be associated with tolerance of cells that produce anti-rat NAb (Aksentijevich *et al.* 1992; Lee *et al.* 1994), in addition to T-cell tolerance. We have more recently demonstrated that B-cell tolerance for the important αGal antigen recognized by human NAb on porcine donors occurs following the induction of mixed chimerism in α1-3Gal transferase (GalT) knockout hosts receiving αGal-expressing BM from wild-type allogeneic donors (Yang *et al.* 1998; Ohdan *et al.* 1999). This tolerization applies to both pre-existing B cells (Ohdan *et al.* 1999), which are not eliminated by the conditioning regimen (Ohdan *et al.* 2000*a*), and to B cells developing after the BMT (Yang *et al.* 1998; Ohdan *et al.* 1999). Antibody-mediated rejection of αGal-positive cardiac allografts is prevented, as is cellular rejection, in these mixed chimeras (Ohdan *et al.* 1999). More recently, we have shown that the anti-αGal NAb barrier to xenogeneic marrow engraftment can be overcome by administering higher than usual rat marrow doses to GalT knockout mice, and that the induction of mixed xenogeneic chimerism in this manner simultaneously prevents hyperacute rejection, acute vascular rejection and cell-mediated rejection of primarily vascularized cardiac xenografts (Ohdan *et al.* 2001). Using a fluorochrome-labelled αGal polymer combined with ELISPOT assays, we have been able to identify and phenotype anti-Gal-producing cell populations in GalT knockout mice, and to assess whether or not they are present in tolerant mice. These studies show that anti-Gal IgM NAb is produced primarily by a CD5-negative and Mac1-negative, but otherwise Blb-like B-cell population in the spleen. Although anti-Gal surface-Ig-bearing cells are present in the peritoneal cavity in larger numbers than in the spleen, the peritoneal cavity B cells do not produce antibody unless stimulated with lipopolysaccharide for several days *in vitro* (Ohdan *et al.* 2000*b*). Mixed allogeneic and xenogeneic chimeras produced in GalT knockout mice show an absence of anti-Gal surface-Ig-bearing cells in the spleen, along with tolerance in ELISPOT assays (Ohdan *et al.* 1999, 2001). However, under certain conditions, anti-Gal surface-Ig-bearing cells may persist in the peritoneal cavity (Ohdan *et al.* 1999). Together, these data suggest that mixed chimerism

leads to tolerization of newly developing NAb-producing cells in the marrow, by either receptor editing or clonal deletion, but that pre-existing anti-Gal-producing cells may be tolerized by other mechanisms. We are now actively investigating the mechanism of tolerization of pre-existing anti-Gal-producing cells, as well as the interrelationship between the apparently anergic peritoneal cavity anti-Gal surface-Ig-bearing cells, which have a more typical Bl cell (Mac-1$^+$) phenotype, and the Mac1$^-$ actively NAb-producing cell population in the spleen.

5. PHYSIOLOGICAL BARRIERS TO ALLOGENEIC AND XENOGENEIC HAEMOPOIETIC CELL ENGRAFTMENT

The concept that 'space' must be created in the haemopoietic compartment in order to allow donor stem cells to engraft has long been widely accepted. In a syngeneic BMT system in which donors and hosts differed only by non-immunogenic alleles of the leucocyte common antigen Ly-5, a low dose (1.5–3.0 Gy) of TBI was required to make physiological 'space' for engraftment of syngeneic marrow cells given in numbers similar to those which could be obtained from marrow of living human allogeneic marrow donors (Tomita *et al.* 1994*c*). However, this requirement can be overcome by the administration of very high doses of syngeneic marrow (Ramshaw *et al.* 1995; Sykes *et al.* 1998). These data are consistent with the possibility that niches for the engraftment of administered stem cells are filled by mass action, and that the effect of TBI is to empty some of the niches, shifting the equilibrium in favour of administered cells. On the other hand, such irradiation appears not to be required if the donor inoculum is sufficiently large and adequate host T-cell depletion is achieved prior to transplantation. The dose of TBI required to achieve allogeneic marrow engraftment in mice can be reduced by administering increasing numbers of donor marrow cells (Bachar-Lustig *et al.* 1995). We have recently demonstrated that engraftment of high doses of allogeneic marrow can be achieved without myelosuppressive treatment in mice that receive T-cell-depleting mAbs (Sykes *et al.* 1997). However, we have learned from these studies that it is essential to create 'space' in the thymus and to achieve high levels of early donor T-cell repopulation in order to achieve permanent skin graft tolerance. Apparently, thymic 'space' and peripheral haemopoietic 'space' are regulated independently, and the stem cell pools contributing to long-term thymopoiesis and haemopoiesis of all other lineages (including B cells) are non-identical (Sykes *et al.* 1998). We have more recently used co-stimulatory blockade to avoid the requirement for TI and for recipient T-cell depletion to achieve lasting mixed chimerism using high doses of donor marrow (figure 2) (Wekerle *et al.* 2000). Lasting engraftment with high-dose marrow administration has also been observed in mice treated with repeated anti-CD40L injections (Durham *et al.* 2000) or with anti-lymphocyte serum and rapamycin (Hale *et al.* 2000). The achievement in humans of successful engraftment of large numbers of T-cell-depleted HLA-mismatched mobilized peripheral blood and marrow stem cells supports the concept that allo-engraftment can also be enhanced in humans by increasing the number of haemopoietic progenitors administered (Aversa *et al.* 1994).

Using peripheral blood stem cells (PBSCs) collected after cytokine mobilization, or using cadaveric donors, it may be possible to transplant large numbers of allogeneic haemopoietic cells to humans. Advances in the ability to propagate primitive haemopoietic cells *in vitro* using cytokine combinations (Petzer *et al.* 1996; Emerson 1996; Brandt *et al.* 1998) and to reconstitute autologous haemopoiesis in patients with progenitor cells expanded *in vitro* (Brugger *et al.* 1995), suggest that *ex vivo* expansion of allogeneic haemopoietic cells could have clinical usefulness. The administration of large numbers of haemopoietic cells, which might be available from inbred porcine donors, is being explored in order to overcome the competitive disadvantage of xenogeneic marrow compared with host marrow (Gritsch & Sykes 1996*b*; Sharabi *et al.* 1990; Gritsch *et al.* 1994; Buhler *et al.* 2000*b*) (see § 6(d)(iv)).

The mechanism by which myelosuppression promotes marrow engraftment is not fully understood, and could include both the creation of physical niches due to the destruction of host haemopoietic cells, and the up-regulation of cytokines that promote haemopoiesis. Homing to the marrow environment depends on interactions between adhesion molecules and their ligands (Papayannopoulou *et al.* 1995; Simon *et al.* 1999), and active haemopoiesis also depends on specific molecular interactions, including cytokines, chemokines and adhesion molecules, between the stroma and haemopoietic cells (Levesque *et al.* 1995, 1996; Verfaillie *et al.* 1991; Voura *et al.* 1997; Schick *et al.* 1998; Goltry & Patel 1997; Kovach *et al.* 1995; Peled *et al.* 1999, 2000; Van der Loo *et al.* 1998; Aiuti *et al.* 1997). The species specificity of some of these interactions accounts, at least partially, for the competitive advantage enjoyed by recipient marrow over xenogeneic donor marrow, as is discussed in the ensuing paragraphs.

In the xenogeneic BMT model presented in figure 3, large numbers of DBMCs are needed to achieve engraftment, and the level of rat haemopoietic reconstitution gradually declines over time, despite persistent tolerance (Sharabi *et al.* 1990; Lee *et al.* 1995). This decline, which can be averted by the late administration of repeated marrow injections (Lee *et al.* 1995), is due to a competitive advantage enjoyed by host haemopoietic cells over xenogeneic cells that becomes increasingly evident as recovery of the host from low-dose TBI occurs (Gritsch & Sykes 1996*a*).

Achievement of xenogeneic haemopoietic repopulation has proved to be an even more formidable challenge in more disparate species combinations. Human and pig progenitor cells have been clearly shown to be capable of repopulating murine recipients at low levels (Pallavicini *et al.* 1992; Lapidot *et al.* 1992; Gritsch *et al.* 1994), but the species specificity of critical regulatory molecules may limit the level of donor repopulation. Administration of exogenous donor species-specific cytokines can partially overcome this barrier (Lapidot *et al.* 1992; Yang *et al.* 1996), and studies with porcine cytokine transgenic mice demonstrate the impressive capacity of high levels of donor cytokines to enhance donor stem cell engraftment

and haemopoiesis, and to even permit the spontaneous appearance, long term, of class-II-positive dendritic cells or macrophages in the murine host thymus (Yang *et al.* 2000; Chen *et al.* 2000). These cell types are of importance in inducing central tolerance of developing thymocytes (Inaba *et al.* 1991; Matzinger & Guerder 1989; Brocker *et al.* 1997) and the presence of such donor cells in the thymus across such a disparate species barrier is highly encouraging for the potential of this approach to induce deletional tolerance. Evidence implicates clonal deletion in the long-term tolerance induced in mixed chimeras prepared in the rat→mouse species combination (Ildstad *et al.* 1992; Tomita *et al.* 1994*b*; Nikolic *et al.* 1998).

6. THE CHIMERISM APPROACH IN LARGE ANIMALS

(a) *The need for large animal models*

Because the non-myeloablative preparative regimen used in mice and shown in figure 1 was far less toxic than the lethal preparative regimen used in previous studies, we have considered it for potential clinical applicability. However, although murine studies have potential for application to the clinical situation, results obtained in rodent models cannot always be simply extrapolated to man. This is due in part to significant differences that exist between rodents and large animals with respect to BMT biology. For example, the preparative regimen by which chimerism was originally quite safely achieved in mice is far too toxic to be used in human beings. Mice were ablated by irradiation with 10.0 Gy delivered at *ca.* 1.0 Gy min^{-1}, and surprisingly, the only organ system that appeared to have been irreversibly damaged was the lymphohaemopoietic system (Bond *et al.* 1965). In contrast, administration of this dose and dose rate of irradiation to human beings and other large animals leads to such irreparable damage in other organ systems, including the gut and lungs, that it cannot be used as a clinical ablative regimen. Consequently, less-toxic ablative regimens have been employed in leukaemic patients needing BMT, including fractionated radiation and use of radiomimetic drugs (Van Bekkum 1984). This difference in preparative regimens may explain some of the discrepancies that have been encountered in trying to extrapolate mouse data to human protocols. For example, T-cell-depleted marrow engrafts readily in lethally irradiated mice even across MHC barriers (Soderling *et al.* 1985; Vallera *et al.* 1981), while similar depletions of human BM have led to a markedly increased risk of engraftment failure (Kernan *et al.* 1987; O'Reilly *et al.* 1985). In addition to species differences of this nature, another obstacle to the extension of murine models to humans is that it is often difficult to reproduce in humans the precise conditions that are used to achieve BM engraftment and tolerance in mice. For example, administration of mAbs at doses sufficient to induce similarly extensive T-cell depletion to that achieved in the murine model in figure 1 has not been achieved in humans. Thus, before attempting to use non-myeloablative BMT approaches for the induction of tolerance in humans, it is considered important to determine whether such regimens used can be applied to large animals. In this laboratory, we have developed two such models, involving miniature swine and cynomolgus monkeys, both of which will be described here.

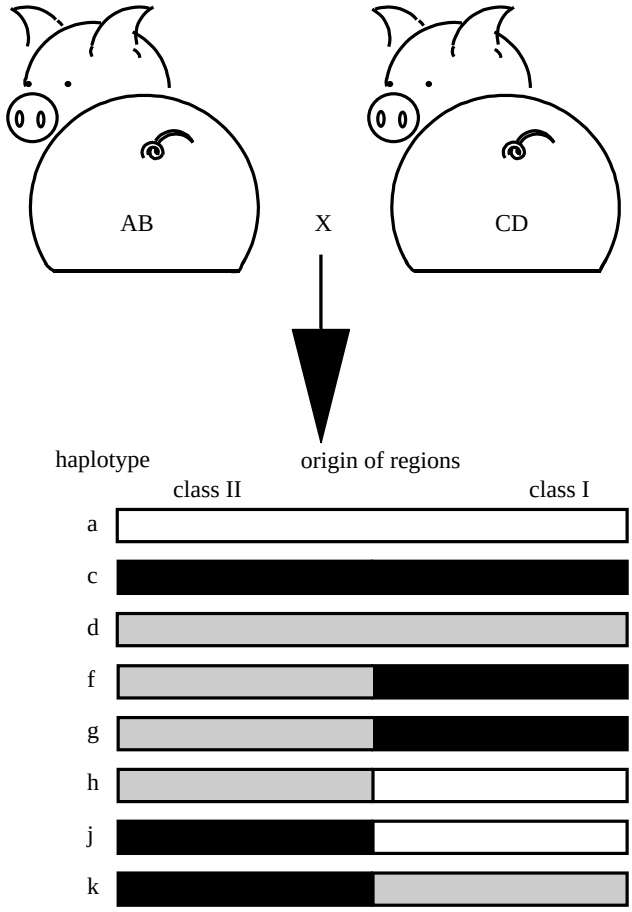

Figure 4. Origin of MGH miniature swine haplotypes.

(b) *Miniature swine*

Over the past 28 years, we have used a selective breeding programme in order to develop and maintain miniature swine with defined MHC genes as a large animal model for studies of transplantation biology (Sachs *et al.* 1976). At present, we maintain swine of three different MHC haplotypes, SLA^a, SLA^c, and SLA^d. We have also developed five lines representing four different intra-MHC recombinant haplotypes, which were derived by spontaneous recombination events during the routine breeding of heterozygotes as part of the breeding programme (figure 4). All of these lines have intentionally not been further inbred for background genes, so that they differ by at least several minor histocompatibility loci. As such, they provide a model in which most of the transplantation combinations relevant to human transplantation can be performed: Transplants within a herd simulate transplants between HLA-identical siblings; transplants between completely MHC-disparate herds resemble cadaveric or non-matched sibling transplants; and transplants between single-haplotype-mismatched heterozygotes (e.g. $SLA^{ac} \rightarrow SLA^{cd}$) resemble parent into offspring or one haplotype-mismatched sibling transplants.

We have reported effective protocols for autologous and allogeneic BMT using this miniature swine model. Using

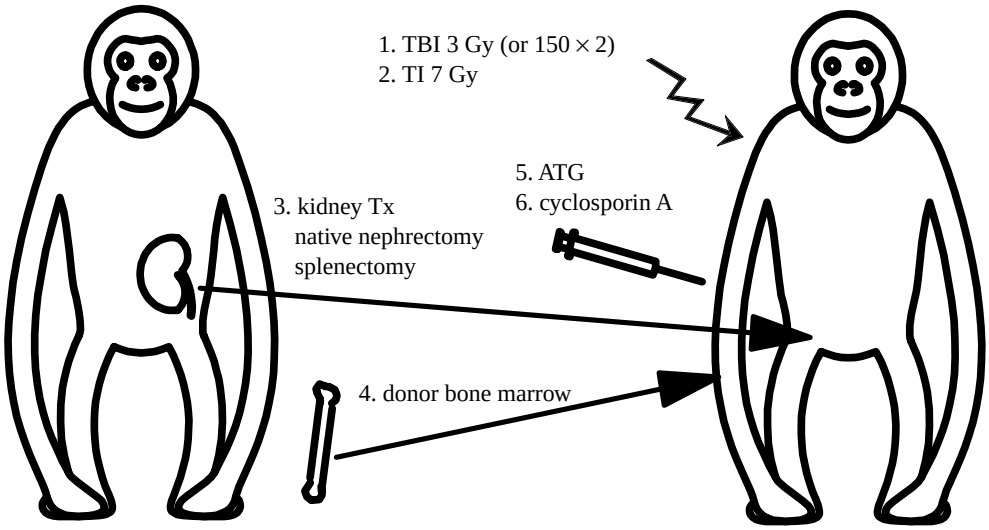

Figure 5. Schematic of protocol for non-myeloablative preparative regimen to induce tolerance between full MHC haplotype-mismatched cynomolgus monkeys. Tx stands for transplant.

a preparative regimen of 9.5 Gy single-dose TBI from a cobalt source, engraftment and long-term survival can routinely be achieved in recipients of autologous or of MHC-matched allogeneic marrow (Popitz-Bergez *et al.* 1988), similar to results in the clinical setting. This preparative regimen was also effective in permitting engraftment in parent into F1 (P→F1) combinations (Popitz-Bergez *et al.* 1988), although such transplants produced severe GVHD. BMT across MHC barriers in pigs has proved much more problematic. Using fractionated high-dose TBI as the preparative regimen, engraftment has been achieved in completely MHC-mismatched donor-recipient pairs. However, severe GVHD developed in such animals, and alloengraftment could not be achieved when inocula were depleted of CD4 plus CD8 T cells, so that the animals died of BM aplasia (Gress *et al.* 1987). In the case of single-haplotype fully MHC-mismatched transplants, the GVHD, although severe, was sometimes self-limited, and stable chimeras were obtained (Guzzetta *et al.* 1991). However, for two-haplotype fully MHC-disparate transplantation, no long-term survivors were achieved.

An effective protocol for inducing mixed chimerism in miniature swine has only recently been made possible by the development of the 2-6-15 monoclonal anti-pig CD3 antibody (Huang *et al.* 1999*a*). In collaboration with Dr David Neville, US National Institutes of Health, we conjugated purified 2-6-15 to the same mutant diphtheria toxin (CRM9) described by Neville and colleagues (Ma *et al.* 1996; Knechtle *et al.* 1997) for an anti-rhesus-monkey CD3 antibody and an anti-human CD3 antibody. This conjugate (pCD3-CRM9) was capable of depleting T cells both from peripheral blood lymphocytes (PBLs) and from lymph nodes of miniature swine to levels of less than 2.0% (Huang *et al.* 1999*b*). Initial attempts to establish mixed chimerism in miniature swine using this reagent for T-cell depletion were performed using BM as the donor stem cell source. However, we have also recently developed methodology for producing huge numbers of cytokine-mobilized peripheral blood enriched for haemopoietic PBSCs in miniature swine (Colby *et al.* 2000; Huang *et al.* 2000). Using these cells as a source of donor haemopoietic cells for transplantation has provided two major advantages over BM as the donor-cell source: (i) the donor can be kept alive for future skin or organ transplantation into the recipient to test tolerance induction; and (ii) the very large numbers of donor haemopoietic cells that can be collected by multiple leukapheresis procedures have made possible for the first time in a large animal model the use of 'megadose' stem cell transplantation, capable of avoiding the need for TBI (Fuchimoto *et al.* 2000).

Using PBSCs and a non-myeloablative preparative regimen based on the one shown in figure 1, consisting of 3 Gy TBI, 7 Gy TI and *in vivo* T-cell depletion with the pCD3-CRM9 immunotoxin, mixed chimerism was achieved between swine leucocyte antigen (SLA)-matched, minor antigen-mismatched animals, as detected by FCM in the peripheral blood, thymus and BM. This protocol produced stable mixed chimerism without any clinical evidence of GVHD, and recipients were rendered specifically tolerant to the donors as demonstrated by prolonged acceptance of donor skin and/or kidneys and hearts (Schwarze *et al.* 2000; Storb *et al.* 1999). This study thus demonstrated that a relatively non-toxic regimen could be used to achieve stable multilineage mixed chimerism and donor-specific tolerance without chronic immunosuppression or GVHD in this large animal model.

In view of results described in the murine models above (Sykes *et al.* 1997; Wekerle *et al.* 2000), and in an attempt to give this approach greater clinical applicability, we have subsequently attempted to avoid the need for TBI entirely (Fuchimoto *et al.* 2000). Thus, even the non-myeloablative conditioning regimen described above included a low level of TBI, thought to facilitate engraftment by either making 'room' for donor haemopoietic stem cells or by providing sufficient host immunosuppression to enable donor cells to engraft (Storb *et al.* 1999). In our recent studies, we have established mixed chimerism in miniature swine across both minor and major histocompatibility barriers without TBI, by using very high doses of PBSCs. Following the

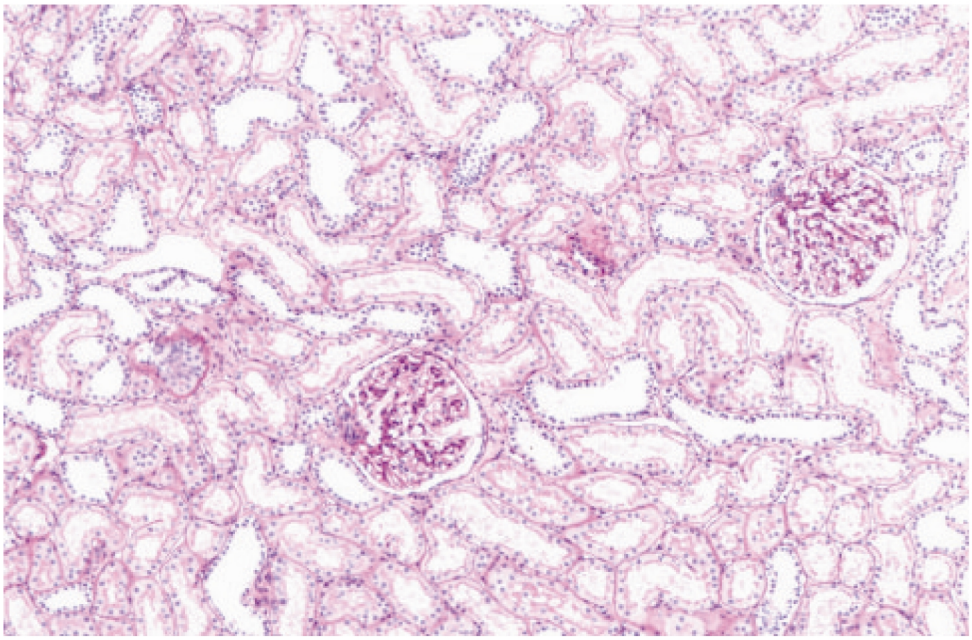

Figure 6. Renal allograft biopsy from animal treated by mixed chimera protocol, taken on day 1912 post-transplantation, demonstrating normal histology.

establishment of mixed chimerism by this procedure, tolerance was confirmed in SLA-matched mixed chimeras by marked prolongation of donor skin grafts compared with prompt rejection of third-party SLA-matched skin. Tolerance was also confirmed in SLA-mismatched mixed chimeras by long-term acceptance of donor-matched kidney allografts without any additional immunosuppression (Fuchimoto *et al.* 2000).

(c) *Cynomolgus monkeys*

We have also developed a non-myeloablative protocol to induce mixed chimerism in cynomolgus monkeys (Kawai *et al.* 1995). Pairs of animals were selected following serological and MLR testing to assure that they were fully MHC mismatched. Animals were chosen such that at least one mAb was available that reacted with the donor MHC and not with the recipient, for detection of chimerism by FCM post-transplantation. As illustrated in figure 5, animals received 3.0 Gy TBI, 7.0 Gy TI and horse anti-human ATG pre-operatively. Bilateral nephrectomy, splenectomy, orthotopic kidney transplantation and donor BM administration were all performed on day 0 (see below for further details on the preparative regimen). In order to supplement suppression of mature T cells by ATG, treatment with cyclosporin A (CyA) intramuscularly was begun on day 1 and continued for four weeks. No further immunosuppression was administered after this time. This protocol was thus highly analogous to that used in our previous murine studies (Sharabi & Sachs 1989).

We have called this regimen, which was found initially to be effective in inducing tolerance, our 'standard regimen'. Of 13 recipients undergoing this conditioning, 11 developed mixed chimerism, and 10 survived long term without allograft rejection. In these long-term survivors, alloantibody and chronic vasculopathy have never been detected, except in one monkey that did not develop clear macrochimerism. Furthermore, all long-term survivors showed donor-specific unresponsiveness in MLRs (Kawai *et al.* 1995). The longest surviving tolerant animal is now more than 2200 days post-transplantation, with a serum creatinine of $1.2\,\mathrm{mg\,dl^{-1}}$ and a blood urea nitrogen of $19\,\mathrm{mg\,dl^{-1}}$; a renal biopsy at 5.5 years post-transplantation showed no abnormality (figure 6). Ureteral stenosis, which has occurred in several recipients at variable time-points post-transplantation (Kawai *et al.* 1995; Kimikawa *et al.* 1997), has been found to be associated with a polyomavirus infection, readily demonstrable by immunohistochemical techniques in the kidney and ureter of affected animals (Van Gorder *et al.* 1999). Multiple modifications of this regimen using either reduced doses of TBI, no TI, or no CyA, all failed to induce chimerism, and all allografts were rejected (Kawai *et al.* 1995; Kimikawa *et al.* 1997).

We have also evaluated the necessity for splenectomy in the regimen, by attempting the standard regimen but without splenectomy in three monkeys. Despite successful induction of chimerism, all three eventually produced alloantibody and the kidney allografts were rejected with evidence of acute and chronic humoral rejection (vasculopathy). We concluded that, if splenectomy is not included, induction of B-cell tolerance does not occur, even in recipients demonstrating macrochimerism (Kawai *et al.* 1999).

In an effort to increase T-cell depletion, we replaced ATG in the regimen with an anti-CD2 mAb (LoCD2, $1\,\mathrm{mg\,kg^{-1}day^{-1}}\times3$) in ten recipients. Although T-cell depletion, measured by FCM, was improved, no monkeys in the LoCD2 treatment group developed chimerism. Five died of infection, and five suffered progressive rejection, with only one recipient surviving beyond 100 days. In the LoCD2 group, despite better depletion of $CD3^+$ T cells, there was less depletion of NK cells ($CD16^+CD3^-$), and NK function quickly recovered by day 5. In contrast, in the ATG group, NK cell numbers were more significantly depleted and NK function remained significantly abrogated for three weeks. We

therefore inferred that there might be an association between NK function and the induction of mixed chimerism and tolerance (Kawai *et al.* 2000*b*).

In order to determine whether the infusion of DBMCs was essential for tolerance induction, we performed experiments in which either no DBMCs were administered or in which donor splenocytes were substituted for DBMCs. All other elements of the conditioning were unchanged. Two monkeys that received no DBMCs rejected their allografts soon after discontinuation of CyA, with no detectable chimerism. Of three monkeys that received donor splenocytes instead of DBMCs, two developed alloantibodies and rejected the kidney. Alloantibody was not detectable in the third monkey, but it died on day 131 from acute humoral rejection. We concluded that the engraftment of donor haemopoietic cells followed by detectable chimerism was essential for tolerance induction in this model.

In order to clarify whether implantation of the renal graft at the time of recipient conditioning was also essential to the induction of tolerance, we have studied ten monkeys which were subjected to standard conditioning, but which did not simultaneously receive a renal transplant. These monkeys subsequently received a kidney (from the BM donor) at various times after conditioning, but without further immunosuppression. Our data indicated that immediate transplantation of the kidney at the time of recipient conditioning was not essential for induction of donor-specific hyporesponsiveness, as kidney allografts transplanted as late as three months after initial conditioning, when chimerism was no longer detectable, were sometimes still accepted (Kawai *et al.* 1999). It seems possible that the presence of the kidney transplant helps to maintain tolerance at later time-points, possibly via peripheral mechanisms (Kawai *et al.* 1995). Further studies will be required to clarify this issue, since some monkeys failed to develop chimerism, produced anti-donor alloantibody, and rejected the kidney allograft. As with the standard regimen, splenectomy at the time of DBMC administration (day 0) proved to be essential. In recipients without splenectomy, development of anti-donor antibodies led to acute, or even hyperacute, rejection.

In vitro studies of the longest survivor of these delayed renal transplantation experiments showed a specific hyporesponsiveness to donor antigens, which developed about two months after organ transplantation and which persisted while MLR to third-party cells returned to pretreatment levels. While anti-donor cytotoxic T-lymphocyte (CTL) activity was readily detected at the time of the kidney transplantation (at three months), specific loss of anti-donor CTL activity was apparent by the sixth month. The loss of CTL over this period probably reflected a reduced frequency of precursor CTLs (pCTLs) specific for donor antigens relative to those for third-party antigens. When the sensitivity of the cell-mediated lympholysis (CML) assay was increased by using expanded T-cell lines derived from six-day MLRs, we found a complete inability to generate donor-reactive CTLs by 14 months post-therapy. The results from this and other long-term survivors suggest that the current protocol leads to the eventual deletion of donor-reactive pCTLs. Anergy is a less likely explanation, since CTL stimulations were performed in the presence of IL-2.

Based on the murine studies showing that the addition of co-stimulatory blockade could allow the induction of lasting chimerism and tolerance in mice receiving 3 Gy of TBI and an inadequate amount of T-cell-depleting mAb as conditioning (Wekerle *et al.* 1999), we have recently begun to assess the potential for use of co-stimulatory blockade as part of the conditioning regimen for inducing mixed chimerism in primates. For this purpose, monkeys received a modified regimen including the addition of anti-CD40L mAb to the preparative regimen. This addition improved the resulting levels of chimerism and also eliminated the need for splenectomy (Kawai *et al.* 1999). We also tested whether CyA could be eliminated from the protocol if anti-CD40L was used, but found that this modification led to loss of chimerism and rejection of kidney transplants, as well as to development of anti-donor alloantibodies. Animals treated similarly, but with CyA, developed chimerism, and appeared tolerant. Thus, these preliminary data suggest that the addition of anti-CD40L antibody can improve the reliability of engraftment and tolerance induction. It should be noted that, unlike the studies of Kirk *et al.* (1999), in which the addition of calcineurin inhibition with FK506 appeared to have a negative effect, in our studies, CyA appears to be helpful for the reliable induction of tolerance. These observations presumably reflect the different mechanisms involved in these two approaches.

Despite the evidence that chimerism is essential to the induction of tolerance in this regimen, persistence of tolerance after loss of chimerism suggests that once established, tolerance can be maintained by the presence of the renal allograft. It seems possible that transient chimerism leads initially to central deletional tolerance, as has been demonstrated in our previous mouse studies (Tomita *et al.* 1994*a*), but that unlike the mice, the primates develop insufficient engraftment of haemopoietic stem cells to maintain this form of tolerance permanently. However, new T cells emerging after the loss of chimerism may be more readily susceptible to induction of a peripheral form of tolerance (possibly induced by the presence of the renal allograft) than are mature T cells in a normal animal, so that tolerance to the transplanted organ remains even after the loss of chimerism. Ongoing studies of mechanisms should help us to evaluate this hypothesis.

(d) *Mixed chimerism in a pig→primate discordant xenograft combination*

The approach we have chosen for pig-to-primate transplantation involves extension of the mixed chimerism approach we have used to induce allogeneic transplantation tolerance in monkeys (Kawai *et al.* 1995; and see above (§ 6(c)) to this xenogeneic combination (figure 7). We have previously demonstrated the efficacy of this mixed chimerism approach in producing long-term survival of xenografts in the concordant rat→mouse combination, with the only major difference from the allogeneic mouse protocol being the addition of NK-cell- and γδ T-cell-depleting mAbs to the pretransplantation depletion regimen (Sharabi *et al.* 1990; Nikolic *et al.* 2001).

The great disparity between species as phylogenetically distant as swine and humans would be expected to

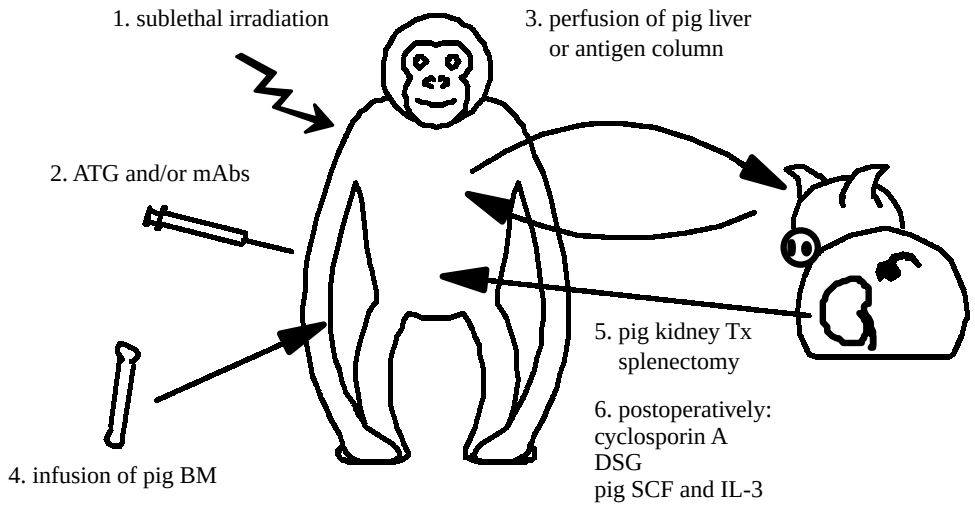

Figure 7. Schematic of non-myeloablative protocol for attempts to induce transplantation tolerance across the discordant xenogeneic barrier pig→cynomolgus monkey through establishment of mixed chimerism. Abbreviations: Tx, transplant; DSG, desoxyspergualin; SCF, stem cell factor.

increase the immunological barrier to xenotransplantation. Therefore, in order to extend our non-myeloablative protocol for inducing tolerance to this discordant system, the following obstacles must be overcome.

(i) *NAbs*

All humans and Old World primates have considerable NAb activity against swine cells, predominantly IgM, but also IgG (Latinne *et al.* 1994; Platt *et al.* 1991). The titres of these antibodies vary from primate to primate, and the reactivity of each serum on cells from different swine is also variable. Because NAbs have been associated with hyperacute rejection of vascularized xenograft organs (Auchincloss 1988; Lexer *et al.* 1986), and because such antibodies would be expected to interfere with BMC engraftment (Aksentijevich *et al.* 1991; Latinne *et al.* 1994), methods for removing these antibodies are required as part of a xenotransplantation approach. Indeed, the removal and inhibition of resynthesis of NAbs is one of the most important near-term goals of xenotransplantation research.

One means for removing NAbs is absorption, and this is the methodology we have used to date in our pig→baboon transplantation model (Xu *et al.* 1998; Sablinski *et al.* 1997). We have made use of the fact that the vast majority of primate anti-pig NAbs are directed at the α(1,3)Gal epitope (Cooper *et al.* 1993*a*,*b*; Galili *et al.* 1984; Oriol *et al.* 1993; Sandrin *et al.* 1993), an important feature of NAbs. We have used columns bearing sugars with this linkage for clearance of NAbs and have thereby prevented hyperacute rejection of pig kidney xenografts (Latinne *et al.* 1993; Sablinski *et al.* 1997; Kozlowski *et al.* 1999*b*). However, subsequent return of these antibodies resulted in the loss of the kidneys. It is for this reason that additional approaches, including mixed chimerism (see § 4(c)) and the use of organs from animals transgenic for complement regulatory proteins (Cozzi *et al.* 1997), may be required for prevention of antibody-mediated rejection.

(ii) *Depletion of mature T cells*

It is clear from rodent studies that complete depletion of mature T cells from the recipient is essential in order to achieve mixed chimerism (Drugan *et al.* 1989; Sharabi *et al.* 1990). In the case of xenografts, depletion of NK cells also appears necessary for success (Sharabi *et al.* 1990; Nikolic *et al.* 2001). We have therefore assumed that similar depletion of T cells and NK cells will be needed in order to achieve mixed chimerism in a discordant xenogeneic model.

Unfortunately, as noted in the paragraphs above, there are very few available mAbs to mature primate T-cell subsets and NK cells that are capable of depleting *in vivo*. Even those that are depleting do not deplete completely, as evidenced by persistence of T cells coated with antibody in the peripheral circulation and especially in lymphoid tissues (Wee *et al.* 1992; Kawai *et al.* 1995). There is likewise no good depleting anti-NK-cell mAb available for use in primates. Therefore, we have undertaken a programme to develop appropriate depleting mAbs and are continuing to screen antibodies from other sources as available. Although such mAbs would have the major advantage of reproducibility, we have proceeded with our studies using a polyclonal anti-thymocyte globulin (ATG) (Upjohn, Kalamazoo, MI, USA). We have demonstrated efficacy of this reagent in eliminating mature T cells and NK cells from the peripheral blood by fluorescence-activated cell sorting (FACS) analyses with appropriate mAbs and by functional assays of T-cell and NK cell activity (Latinne *et al.* 1993). Even this ATG preparation, however, has not been capable of eliminating all mature T cells when used *in vivo*. While all peripheral T cells in PBLs disappeared by one to two days after *in vivo* treatment, viable T cells coated with ATG have been found in the lymph nodes at these times (Kawai *et al.* 1995). Therefore, in order to inhibit T-cell function during the immediate post-transplantation period, and hopefully permit engraftment of pig BMCs, we have added treatment with CyA for one month post-transplantation to the regimen. Since none of our pig→monkey xenografts has yet survived beyond 29 days, it is unclear at present whether or not the CyA has been effective for this purpose. However, in the related mismatched monkey→monkey allotransplantation protocol, treatment

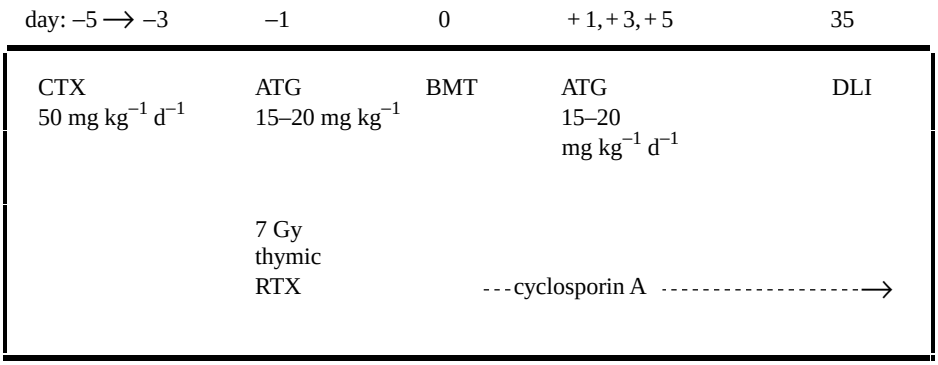

Figure 8. Protocol for induction of mixed chimerism in a clinical trial in patients with refractory haematological malignancies (Spitzer *et al*. 1999*b*; Sykes *et al*. 1999). CTX stands for cyclophosphamide.

with ATG and CyA, especially with the addition of a short postoperative course of anti-CD40L, has permitted engraftment of fully allogeneic BM and the induction of tolerance (Kawai *et al*. 1995, 1999, 2000*b*). We are therefore hopeful that this level of T-cell depletion or inhibition should be sufficient to permit engraftment in the xenogeneic model as well.

(iii) *Myelosuppressive regimen*

In both the murine allogeneic and mouse rat concordant xenogeneic tolerization protocols, it was necessary to make 'room' for the donor BM to engraft by administration of a sublethal dose of TBI (Sharabi & Sachs 1989; Sharabi *et al*. 1990). In addition, because T cells were found in the thymus, coated with antibody but not eliminated, following administration of anti-T-cell mAbs, an additional boost of irradiation to the thymus was added to the regimen in order to achieve mixed chimerism. Thus, in the mouse, a dose of 3.0 Gy of TBI plus 7.0 Gy of TI was used to achieve mixed chimerism. We therefore adapted the same irradiation protocol to cynomolgus monkeys for our allogeneic mixed chimerism studies (Kawai *et al*. 1995), as well as for our initial attempts to induce xenogeneic mixed chimerism in the pig→monkey system.

(iv) *Pig haemopoietic cell engraftment*

Rodent studies have shown an excellent correlation between the achievement of mixed chimerism and the induction of long-term transplantation tolerance (Sharabi & Sachs 1989). Our recent attempts to increase engraftment of xenogeneic pig BM in non-human primates have used pig cytokines (Sablinski *et al*. 1999; Kozlowski *et al*. 1999*a*), co-stimulatory blockade with anti-CD40L antibody (Buhler *et al*. 2000*a*) and high-dose PBSC infusion (Nash *et al*. 1999; Buhler *et al*. 2000*a*). Evidence for engraftment of pig haemopoietic cells has been obtained for up to three weeks by FACS analyses (i.e. macrochimerism), and for over one year by PCR (i.e. microchimerism) (Kozlowski *et al*. 1999; Buhler *et al*. 2000*a*). However, the kind of long-term macrochimerism associated with T- and B-cell tolerance in mice has not yet been achieved.

(v) *Current status of discordant organ transplantation*

All of these considerations have led to the protocol for pig→monkey renal xenotransplantation illustrated schematically in figure 7. Our initial results using this model have been reported (Latinne *et al*. 1993; Tanaka *et al*. 1994; Sablinski & Sachs 1995; Kozlowski *et al*. 1999, 2000), and have demonstrated feasibility of the approach. Our results to date have shown elimination of hyperacute rejection by the absorption procedure, and kidney graft survival times of up to 29 days. Biopsies during the first few weeks showed essentially normal kidneys. However, long-term survival has been limited by the occurrence of thrombotic and haemorrhagic events, which lead to loss of the kidney. Rejection appears to correlate with the reappearance of IgG anti-swine antibodies, which can also be seen by immunohistology in the rejected organs. Interestingly, in animals treated with our induction protocol, and especially in those receiving anti-CD40L post-operatively, the returning NAbs showed the same specificity for $\alpha(1,3)$Gal epitopes as did the antibodies prior to treatment. In contrast, animals rejecting pig xenografts with only standard immunosuppression make large amounts of antibody to additional determinants on pig cells. Thus, it appears that the induction regimen we are using may have the capacity to induce tolerance at the T-cell level sufficient to inhibit a T-cell-dependent antibody response (Kozlowski *et al*. 1999). Clearly, efforts will have to made to achieve tolerance at the antibody level before long-term engraftment will be possible, and studies to this end are in progress (Alwayn *et al*. 1999). Studies in the murine model suggest that overcoming transplantation barriers sufficiently to allow the initial induction of substantial levels of mixed chimerism could effectively achieve this goal (Yang *et al*. 1998; Ohdan *et al*. 1999, 2000).

7. APPLICATION OF NON-MYELOABLATIVE BMT FOR THE INDUCTION OF TOLERANCE IN HUMANS

A number of years ago, we made the observation in mice that established mixed chimeras that receive infusions of non-tolerant donor T cells convert to full chimerism without developing GVHD (Sykes *et al*. 1988*b*), suggesting a way of separating graft-versus-leukaemia (GVL) effects of alloreactivity from its tendency to cause GVHD. With the goal of exploiting the GVL effect of these delayed donor-leucocyte infusions (DLI) in patients with haematological malignancies who might be too old or otherwise unable to tolerate a conventional BMT, we modified

our original murine non-myeloablative BMT preparative regimen by replacing TBI with cyclophosphamide. DLI was again shown to convert mixed chimeras to full chimeras without causing GVHD (Pelot *et al.* 1999). This model was adapted in a clinical trial with the goal of inducing mixed chimerism, then administering DLI to elicit GVL effects without GVHD in patients with refractory haematological malignancies. Recipient treatment included cyclophosphamide, TI, pre- and post-transplantation ATG (Upjohn), and post-transplantation CyA until the time of DLI on day 35 (figure 8). As in the monkey model described above, CyA is needed post-transplantation because ATG is unable to fully deplete T cells from the recipient or donor, so initial immunosuppression is needed to avoid the effects of their alloreactivity. With this protocol, long-lasting mixed chimerism was induced in a series of HLA-matched donor BMT recipients, and also in a small series of HLA-mismatched BMT recipients (up to two out of six HLA antigen mismatches in the GVH and host-versus-graft directions) (Sykes *et al.* 1999; Spitzer *et al.* 1999*b*). A quarter to a third of these patients have achieved lasting remissions, which is quite striking in view of the advanced, chemorefractory nature of the malignancy in each case. These early results have demonstrated the principle that DLI can convert mixed chimeras to full chimeras without causing severe GVHD, and that this can be associated with powerful GVL effects. Furthermore, we have demonstrated that lasting mixed chimerism can be achieved across substantial HLA barriers in humans without myeloablative host conditioning. Although GVHD has still been a major complication when extensive HLA barriers have been crossed with this BMT protocol (Sykes *et al.* 1999), we are hopeful that this will be avoided in future by using improved reagents for *in vivo* depletion of T cells from the donor marrow in addition to the host, as we have achieved in the mouse models described above (Sharabi & Sachs 1989; Pelot *et al.* 1999). This initial *in vivo* T-cell depletion of donor marrow is essential for the avoidance of GVHD from the marrow transplant, a precondition that must be met before delayed DLI can be administered safely.

The results obtained to date raise hope that an acceptable protocol for use in organ transplant recipients may soon be feasible. We have recently had an opportunity to test this approach to tolerance induction using a modified version of the protocol in figure 8. A simultaneous kidney transplant and BMT from the same HLA-identical living donor was given to a patient with multiple myeloma and consequent renal failure. Her myeloma is in clinical remission and her kidney is functioning normally, despite the fact that she has been off all immunosuppression for more than 20 months (Spitzer *et al.* 1999*a*). This outcome demonstrates the principle that BMT can be safely used with non-myeloablative conditioning for the induction of organ allograft tolerance in humans, and raises hope that, by making use of recent improvements in immunosuppressive agents to deplete and/or inactivate alloreactive T cells in the donor and host, this approach to transplantation tolerance will be widely applied in the organ transplant population in the not-too-distant future.

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